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14. ABSTRACT
The project aimed to unravel the molecular mechanism of structure alteration and force generation of protein actuators called forisomes. The proteins undergo a rapid calcium induced ATP independent anisotropic contraction. We aimed to express recombinant forisome proteins in a cell free wheat germ system and crystallize the proteins for x-ray diffraction and electron microscopy. While expression of the proteins was achieved, the conditions in the wheat germ system prevented crystallization. Therefore we expressed proteins carrying His-tags to purify proteins. Purified proteins are currently subjected to various conditions for crystallization.

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Report Title

Final Report: Forisome Based Smart Materials

ABSTRACT

The project aimed to unravel the molecular mechanism of structure alteration and force generation of protein actuators called forisomes. The proteins undergo a rapid calcium induced ATP independent anisotropic contraction. We aimed to express recombinant forisome proteins in a cell free wheat germ system and crystallize the proteins for x-ray diffraction and electron microscopy. While expression of the proteins was achieved, the conditions in the wheat germ system prevented crystallization. Therefore we expressed proteins carrying His-tags to purify proteins. Purified proteins are currently subjected to various conditions for crystallization.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Sutton Mooney	0.50
FTE Equivalent:	**0.50**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Jan Knoblauch	0.13	Biological Sciences
Adam Dodgen	0.10	Computer Sciences
Alexander Howell	0.04	Biological Sciences
Katelyn Ward	0.04	Biological Sciences
FTE Equivalent:	**0.31**	
Total Number:	**4**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	4.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

Project: Forisome based smart materials

Cloning strategy to express SEO proteins in cell free systems. We hypothesized that SEO protein growth is limited by cell space, and that these protein bodies only grow to a certain size based on physical borders, such as cell membranes and walls. To bypass this problem, we anticipated that expression of SEO proteins in a cell-free system would allow growth of SEO protein bodies. The company Promega offers several *in vitro* transcription/translation coupled systems that allow up to 100µg/ml protein expression in a single reaction. We chose the TnT® SP6 High-Yield Wheat Germ Protein Expression System since this is a plant-based system, and we anticipated that this might provide the best chances to express high levels of SEO proteins for subsequent structural studies.

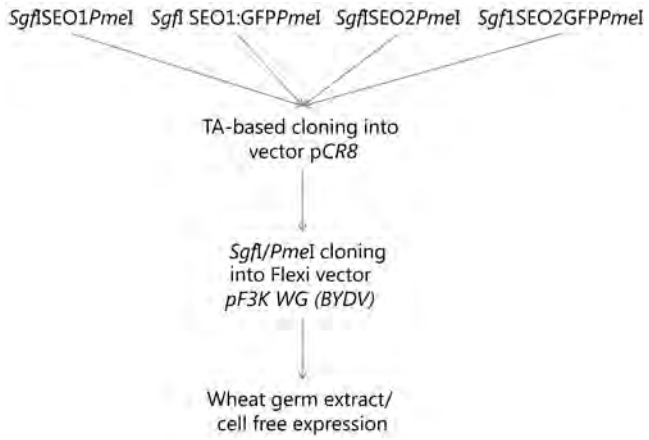

Fig. 1: **Initial cloning strategy of SEO1 and SEO2 for expression in wheat germ extracts.**

We generated constructs from *Medicago truncatula* cDNA that could express *SEO1* and SEO2, either untagged or with a C-terminal GFP. The GFP allowed optical tracking of SEO protein expression through a microscope. For our cloning strategy, *Sgf*I and *Pme*I restriction sites were added at the 5' and 3' ends, respectively, of each PCR product. The products were subcloned via TA-cloning into the vector *pCR8* (Invitrogen), sequenced, and further cloned by classical restriction site based cloning into the *Sgf*I/*Pme*I sites of the Flexi vector *pF3K WG (BYDV)* (for an overview of the cloning strategy see Fig. 1). The Flexi vector was recommended by Promega to yield the highest expression results in the TnT SP6 expression system.

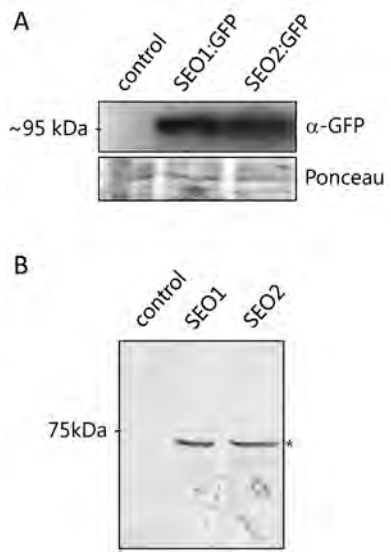

Fig. 2: **Expression of SEO1 and SEO2 in wheat germ extracts. A,** Immunoblot using an α-GFP antibody results in detection of SEO1:GFP and SEO2:GFP at around ~95kDa. **B,** SEO1 and SEO2 detection in reactions where lysine^{GREEN} had been added. In (A) and (B) 1 µl from a 50µl reaction was loaded. Controls represent 2.5 µl of untreated wheat germ extracts.

Expression of SEO proteins in wheat germ extract. We followed two strategies for protein production. 1. We attempted to use a fast production method without protein purification to crystalize in the wheat germ mixture. After generation of the different constructs, we started to optimize the conditions for expressing the two SEO proteins in the wheat germ extracts. Initial factors that needed to be considered were the purity and amount of DNA added to the assay, time of incubation, and RNAse inhibition. We monitored increase of GFP fluorescence by fluorescence microscopy in order to optimize conditions and gained best results by having 3µg plasmid, 1µl RNAse inhibitor (N2511

Promega 40U/ul), incubated in 30µl wheat germ extract at a total volume of 50 µl for 2 hours at 25^0C. SEO expression was easy to detect, and we also detected a single band of the expected size of SEO:GFP proteins (~95 kDa) on Western blot using a specific α-GFP antibody (Fig. 2A). In addition we visualized expression of untagged SEO proteins (~75 kDa) by adding 1 µl Lysine^{Green} (L500A Promega) to the reaction (Fig. 2B). Lysine green is a modified amino acid carrying a fluorescent group, which allows detection of labelled proteins through read out by a phosphoimager after SDS-PAGE.

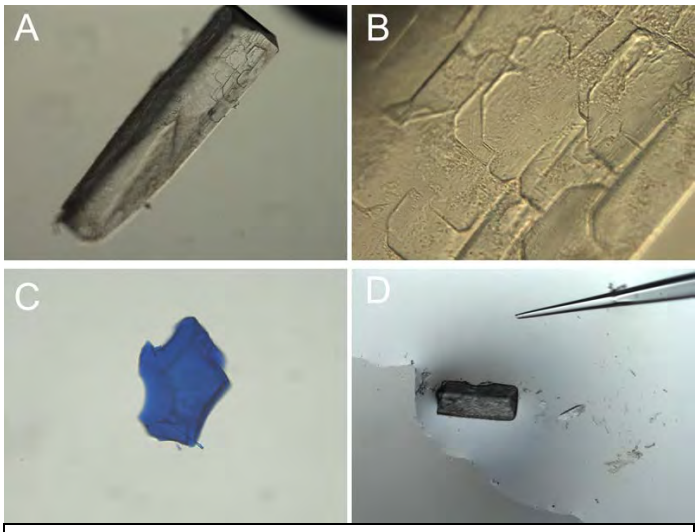

Figure 3: Crystal formation in wheat germ extract. A,B) Crystal formation was observed in wheat germ extract after production of SEO proteins. C,D) fracturing crystals using microcapillaries (D) and staining with coomassie brilliant blue indicated a proteinaceous nature of the crystal.

The results demonstrated that the wheat germ system is suitable to express SEO proteins to full length. We kept solutions under different temperature and humidity conditions to stimulate crystal formation and we were able to generate crystals (Fig. 3). Staing of crystal fragments with coomassie brilliant blue indicated a proteinaceous nature of the crystals. X-ray investigations, however, showed that the crystals were products from components of the wheat germ solution and not SEO protein crystals. Therefore, several additional conditions were tested. We used natural forisomes to dope the solution with a nucleation site unfortunately without success. SEO proteins have 11 cysteines that may form sulfide bonds critical for complex formation. The wheat germ extract contains reducing agents, such as DTT, that may prevent establishment of sulfhydryl-bridges. In addition it is known that SEO-based forisomes loose reactivity over time when exposed to oxygen, while calcium promotes forisomes to go from a low- into a high-volume state. To address these potential problems, Promega recommended adding glutathione to the reaction, and we generated an anaerobic environment by incubating translation reactions in chambers containing BBL™ GasPak™ Plus anaerobic system envelopes with Palladium catalysts. To avoid any potential impacts through calcium we ran reactions in the presence of EGTA. However, these additional treatments

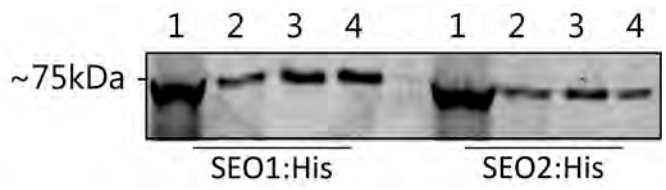

Fig. 4: **Expression and purification of SEO:His tagged proteins.** SEO1:His and SEO2:His were expressed in wheat germ extract as described above in the presence of Lysine^{Green}. Expressed protein was purified through Ni-agarose beads using standard procedures. Proteins were loaded on a SDS-PAGE and detected through a phosphoimager. 1, input (5µl of reaction); 2, first washing (SEO protein unspecifically washed off; all protein loaded), 3, purified and eluted protein (5µl of 100 µl loaded); 4, remaining protein on beads after elution (all protein loaded).

had no positive impacts on forisome assembly, although the same levels of SEO protein expression were observed.

Expression and purification of SEO:His tagged proteins. At this point we speculated that something in the wheat germ extract may be preventing assembly of SEO proteins into forisome complexes. Therefore we changed the strategy and purified SEO proteins from the wheat germ system. *SEO1* and *SEO2* were re-cloned as described above, but with a 6xHis-tag added to the 3'-end of each cDNA for affinity purification. As observed before, we were able to generate full-length SEO proteins, and to purify the proteins from wheat germ extract with Ni-agarose beads (Fig. 3). The purification of the expressed SEO proteins also allowed us to quantify the total amount of SEO proteins expressed in a 50 µl reaction. We repetitively saw production of less than 100 ng. This amount was around 50-fold less than what Promega stated the TnT® SP6 High-Yield Wheat Germ Protein Expression System is capable to produce. Unfortunately the low expression forced us to reduce the total volume for crystallization. However, purified protein is currently subjected to various conditions for slow crystal growth which provides better growth efficiency.

Since the expression in the wheat germ system yielded lower amounts than expected and we cannot exclude that forming crystals will be too small, we have cloned appropriate constructs to use other systems such as yeast expression systems that extract recombinant proteins into the medium. We expect this system to be more efficient, but the final tests will extend beyond the funding period.

Due to some unexpected problems we were not able to crystallize SEO proteins for X-ray investigations within the 9 months funding period. However, we made significant and important progress towards our goal. Since it may take months to form individual crystals under the optimum conditions the proteins are currently subjected to, it is difficult to estimate when crystal formation will be accomplished, but we expect to get diffraction patterns within this year. Once an appropriate publication will be submitted, ARO support will be mentioned and we will upload appropriate publications.